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Amadori glucose adducts modulate mesangial cell growth and collagen gene expression

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Amadori glucose adducts modulate mesangial cell growth and collagen gene expression. Diabetic serum contains increased concentrations of glycated proteins, which are preferentially transported into the renal glomerulus. We investigated effects of Amadori glucose adducts in serum proteins, the predominant form in which circulating glycated proteins exist *in vivo*, on glomerular mesangial cells, where the lesion of diabetic nephropathy originates. [^{3}H]-thymidine incorporation by murine mesangial cells was significantly inhibited when cells were grown in the presence of serum glycated by incubation for four days with 28 mM glucose or when cells were cultured in microtiter plates that had been precoated with glycated serum. This effect was prevented by a monoclonal antibody immunoreactive with Amadori adducts in glycated albumin, and unreactive with other glycated serum proteins or with advanced glycation end (AGE) products. Glycated serum stimulated Type IV collagen gene expression and increased Type IV collagen secretion, an effect also prevented by monoclonal antibodies reactive with Amadori adducts in glycated albumin. The glycation-induced changes in proliferation, collagen synthesis and collagen gene expression were observed in media containing normal glucose concentration and were exaggerated in media containing high glucose concentration. The data indicate that Amadori products of glycated serum proteins induce mesangial cell abnormalities that are highly relevant to the pathogenesis of diabetic nephropathy, and that these effects are accentuated when glycated serum proteins are presented in a hyperglycemic milieu. The data also suggest that mesangial cells specifically recognize Amadori adducts in glycated albumin.

The glomerular mesangial cell is intimately involved in the development of diabetic nephropathy, which is characterized by glomerular hypertrophy, mesangial matrix accumulation in association with decreased cellularity, and a thickened glomerular basement membrane [1–4]. Mesangial cells synthesize Type IV collagen, the predominant constituent of the expanded mesangial matrix that is observed in the diabetic glomerulus, and we and others have used mesangial cell proliferative and biosynthetic responses to media manipulations to investigate potential nephropathic factors [5–11]. High glucose concentration causes delayed growth inhibition in association with bioactivation of transforming growth factor-beta, and induces increased mesangial cell Type IV collagen production and gene expression [6, 9–11]. These findings are interpreted to support a causal role of hyperglycemia in the pathogenesis of diabetic nephropathy. However, responses to high glucose in serum-free media may not satisfactorily address the diabetic milieu *in vivo*, where the mesangium is bathed in serum containing increased concentrations of nonenzymatically glycated serum proteins.

Nonenzymatic glycation (NEG) refers to the condensation between glucose and reactive protein amino groups that results in the formation of stable adducts in Amadori configuration [12–14]. Although subsequent rearrangement reactions may occur, the Amadori adduct is the predominant form in which circulating glycated proteins exist *in vivo*, and its concentration is significantly increased in diabetes with attendant hyperglycemia [2, 13, 15–19]. For example, a recent study describing immunochemical analysis of advanced glycation end (AGE) products in normal and diabetic human serum reports concentrations of 10 to 25 U/ml, equivalent to <0.01% of serum proteins, whereas Amadori products typically constitute 2 to 10% [20]. We became interested in the potential role of NEG of serum proteins in diabetic nephropathy in light of the above, as well as several studies demonstrating adverse effects of glycated serum proteins on glomerular physiology and biochemistry. Glycated serum proteins are preferentially transported across the glomerular filtration barrier, and glomerular mesangial and epithelial cells exhibit enhanced uptake of glycated albumin, which is accompanied by an increase in cell hydrogen peroxide production [21–24]. A most striking finding is the induction of glomerular hyperfiltration, an early functional abnormality implicated in the development of diabetic nephropathy, in normal rats transfused with glycated serum proteins containing Amadori products of the glycation reaction in concentrations similar to those found in streptozotocin-diabetic rats [25].

In approaching study of the influence of serum protein glycation on mesangial cell biology, we considered it important that the glycated proteins be represented as the early (Amadori) glycation products, and therefore adhered to conditions for glycation known to yield Amadori and not AGE modifications [25, 26]. We also examined effects in both normal and elevated media glucose concentration. In this manner, we hoped to delineate glycation-induced from glucose-induced responses, although we anticipated that effects might be additive. Additionally, we used a monoclonal antibody that has been shown to immunoreact with Amadori glucose adducts in albumin and to

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be unreactive with AGE-modified human albumin to identify the contribution of this principle serum protein to glycation-induced abnormalities [27]. We report that exposure of mesangial cells in culture to glycated serum proteins inhibits proliferation and stimulates Type IV collagen gene transcription and production, and that high glucose concentration in the culture media exaggerates these effects. The glycated serum-induced decrease in cell proliferation and increase in collagen production are prevented by monoclonal antibodies reactive with glycated epitopes in albumin, implicating Amadori products of nonenzymatically glycated albumin in the genesis of mesangial abnormalities that are highly relevant to the pathogenesis of diabetic nephropathy.

Methods

Cell culture

Murine mesangial cells in culture were derived from glomeruli isolated from SJL/J mice with a graded sieving technique and plated to explant [28, 29]. Mesangial cells were selected and subcultured according to previously described criteria [28], including morphology, their ability to grow in medium containing D-valine instead of L-valine, and the presence of cytoplasmic filaments (desmin and vimentin), angiotensin II binding capacity, and contractile response to angiotensin II. Experiments examining the influence of glycated serum on mesangial cell proliferation used cells stabilized in long-term culture by virus transformation with SV-40 and carried in DMEM containing 5.5 mM glucose and 10% fetal calf serum (FCS). The suitability of transformed mesangial cells, and their parallelism with non-transformed cells with respect to growth and response to media manipulations, have been documented [11]. Experiments examining the effects of glycated proteins on Type IV collagen production and gene expression used phenotypically stable non-transformed cells grown to 10 to 16 passages in RPMI containing 10 mM glucose and 10% FCS. To initiate experiments under varying culture conditions, mesangial cells were plated into 75 cm^2 plastic flasks or 24- to 96-well plastic microtiter plates, grown to confluence for two days, rested for one day in serum-free medium containing 5.5 mM glucose, and then grown for 48 to 96 hours in fresh DMEM containing 5.5 mM or 25 mM glucose without or with the addition of supplements described below.

Culture conditions

Media supplements consisted of FCS or normal or glycated human serum, and A717 monoclonal antibody or IgG not reactive with glycated albumin. Where indicated for studies on collagen secretion, media also were supplemented with 50 μg/ml each of ascorbic acid and β-aminopropionitrile. For glycation, normal human serum was sterile filtered, incubated for four to five days at 25°C with 28 mM glucose in phosphate buffered saline (PBS), dialyzed against PBS, and again sterile filtered. Unincubated serum also was dialyzed against PBS to control for the effect of dialysis, and sterile filtered. Cell cultures were supplemented to 3 to 10% with FCS, unincubated or glucose-incubated serum, without or with affinity-purified monoclonal antibody A717 or other IgG in PBS (5 to 20 μg/ml final concentration in cell cultures). Production and characterization of the A717 monoclonal antibody have been described [27]. This antibody was raised in mice immunized with glycated albumin purified by affinity chromatography on phenylboronate agarose (PBA) of albumin isolated from human plasma. The immunogen therefore contained Amadori adducts, which bind to PBA, and not AGE products, which do not. The specificity of the A717 monoclonal antibody used in these experiments for glycated epitopes residing in albumin was confirmed with immunoblotting and enzyme-linked immunosorbent assay (ELISA). Antigens tested included nonglycated albumin and nonenzymatically glycated hemoglobin, apolipoprotein B (apo B), fibronectin, and fibronectin auto-digest peptides from 30,000 to 120,000 molecular weight. The glycated species were purified by PBA affinity chromatography from the corresponding proteins isolated from erythrocyte lysates and human plasma, respectively.

Antibody specificity

For immunoblotting, samples were electrophoresed on 12% polyacrylamide gels, and the proteins were transferred electrophoretically to nitrocellulose filters. The transfers were incubated with A717 antibody and developed with an alkaline phosphatase (AP) immunoblot system. ELISA was performed by immobilizing antigen onto microtiter wells in carbonate/bicarbonate coupling buffer, pH 9.5 for four hours at room temperature. Unbound antigen was aspirated and the wells were blocked with 4% milk in glycine coupling buffer, pH 8.5 overnight at 4°C. After washing with 0.15 M NaCl containing 0.01 M triethanolamine, 0.05% Tween 20 and 0.02% Proclin, pH 6.8, 1 μg of monoclonal antibody A717 in 0.1 mM tetrasodium EDTA, 0.17 mM disodium EDTA, 0.15 M NaCl, pH 6.5 was added and allowed to react for two hours at room temperature. After washing with TEA/NaCl/Tween wash buffer, horseradish peroxidase (HRP)-conjugated goat anti-mouse IgG (BioRad, Richmond, California, USA; 1:3000 dilution in 0.15 M saline, 10 mM Tris-acetate, 0.1% milk) was added and incubated for one hour at room temperature. Color was developed with TMBlue substrate and, after stopping with 2 M sulfuric acid, absorbances were read at 450 nm.

Cell growth

[^{3}H]-thymidine incorporation assays were performed in 96-well microtiter plates in 200 μl total volume. At the end of a 48-hour incubation period with unsupplemented or supplemented media, cells were pulsed for eight hours with [^{3}H]-thymidine (1 μCi/well; 5 to 7 Ci/mmol; Amersham, Arlington Heights, Illinois, USA). Cells were collected on glass microfiber filters (934-AH, Whatman, Clifton, New Jersey, USA) using a cell harvester, and radioactivity was assayed after placing the filters in liquid scintillation fluid and counting in a liquid scintillation counter [11, 30]. In parallel studies, mesangial cells were cultured in 24 well plates under similar conditions, the media were then saved for Type IV collagen assay (see below), and harvested cells were counted with an automated cell counter (Coulter Electronics).

RNA isolation and Northern blot analysis

For each experiment, 2×10^6 mesangial cells plated into plastic flasks and cultured as described were used for RNA isolation. Exposure under the different experimental culture conditions was conducted for 72 to 96 hours in order to

maximize cell density and RNA recovery. The cells were lysed and denatured by adding to the flasks 4 M guanidinium thiocyanate, 25 mM sodium citrate (pH 7.0), 0.5% sarcosyl and 0.1 M 2-mercaptoethanol. Total RNA was then extracted by treatment with phenol-chloroform according to Chomcyzynski and Sacchi [31]. Ten to 20 μg of total RNA, assayed by UV spectrophotometry, was electrophoresed through a 1% agarose gel with 2.2 M formaldehyde. RNA transfer to positively charged nylon membrane was performed by electroblotting followed by short-wave UV cross linking and prehybridization as described [32]. Hybridization was conducted for 20 hours at 42°C using excised cDNA inserts labeled with [^{32}P]-dCTP as probes [33]. The cDNA probes used were a 0.65 kb fragment encoding the mouse alpha 1 chain of the Type IV collagen gene and a 1.3 kb Pst I fragment encoding the rat glyceraldehyde-3-phosphate dehydrogenase (GAPDH) gene. After hybridization, the membranes were washed and exposed to x-ray film as described [32]. The filters were first probed with the Type IV collagen cDNA. To account for variations in RNA loading and transfer, the filters subsequently were stripped for one to two hours at 65°C with a solution containing 5 mM Tris, 0.2 mM EDTA (pH 8.0) and 5% sodium pyrophosphate in order to remove the signal. The filters were then rehybridized with the GAPDH cDNA probe.

Type IV collagen secretion

Media were removed at the end of the incubation period and immediately stored at −70°C until use. Type IV collagen was measured by competitive enzyme-linked immunoassay (ELISA) using Type IV collagen from Engelbreth-Holm Swarm tumor as standard, rabbit anti-mouse Type IV collagen as primary antibody (both from Collaborative Research), and HRP-conjugated goat anti-rabbit immunoglobulin as detector (BioRad). Type IV collagen was immobilized onto microtiter plate wells in 0.05 M HCl overnight at 4°C and, after washing, wells were blocked for one hour at room temperature with PBS/10% FCS. Incubation with standard (5 to 500 ng/well diluted in culture media containing 10% FCS) or sample, after addition of primary antibody (1:8000 dilution), was for one hour at room temperature. After washing, wells were incubated with HRP-conjugated anti-rabbit IgG (1:3000 dilution) for 30 minutes, followed by washing, addition of TMBlue substrate (microwell peroxidase system), stopping with 2 M H_2SO_4, and reading absorbances at 450 nm. The assay was sensitive to 5 ng/well.

Protein synthesis

Total protein synthesis in cultured mesangial cells was assessed by tritiated leucine incorporation, and expressed as cpm/10^3 cells as previously described [30].

Results

Previous studies examining the effect of glycated proteins on mesangial cell growth and matrix biosynthesis have used proteins extensively modified by AGE products and crosslinks [7, 34]. These studies found that [^{3}H]-thymidine incorporation was inhibited when cells were plated onto matrix containing fluorescent, cross-linked glycation products obtained after long-term exposure to high concentrations of glycoaldehyde or glucose-6-phosphate (G-6-P). To optimize conditions in this study for the generation of Amadori adducts and minimize AGE-product formation, incubation with glucose was kept to four to five days in the presence of 28 mM glucose, conditions which generate little if any AGE products [25, 26, 35]. For example, the pyrraline AGE compound reported by Miyata and Monnier does not form in albumin incubated with 50 mM glucose until 20 days of incubation [35]. AGE-bovine serum albumin is typically prepared by incubating the protein for four to six weeks with 0.25 to 1.0 M glucose or G-6-P [8, 20, 34]. We sought evidence of AGE-product formation by examining the unincubated and glucose-incubated sera used in this study for fluorescence and by SDS-gel electrophoresis. The two preparations yielded minimal fluorescence at excitation and emission wavelengths of 360 nm and 415 nm, respectively (Table 1) and, compared with unincubated sera, no new high molecular weight cross-linked bands were discerned in the glycated preparations subjected to SDS-polyacrylamide gel electrophoresis on 7.5% or 12% gels under reducing or non-reducing conditions (not shown). These findings support the interpretation that there is not significant AGE product formation after four days of incubation in 28 mM glucose.

Table 1. Fluorescence of normal and glycated human serum

	Unincubated serum	Glycated serum
Fluorescence *arbitrary units/ml*	16	19
Absorbance *280 nm/ml*	1.29	1.29
Protein *mg/ml*	36.0	34.0
Albumin *mg/ml*	27.7	24.9

Fluorescence was determined in a spectrofluorometer using an excitation wavelength of 360 nm, an emission wavelength of 415 nm, and a slit width of 2 mm both for excitation and emission. For comparative purposes, arbitrary fluorescence units/ml in solubilized tissue protein from diabetic rats ranged from 300 to 600, and fluorescence normalized per mg of protein ranged from 150 to 300.

We also showed that the glucose-incubated sera reacted strongly with the A717 monoclonal antibodies, which selectively recognize Amadori adducts in glycated albumin and do not react with nonglycated albumin, AGE-albumin, or other proteins, whether glycated or not. Glucose-incubated serum, which contains many glycated proteins, exhibited only one immunoreactive band showing co-identity with (glycated) albumin when immunoblotted with the A717 antibody (Fig. 1). Glycated hemoglobin (Fig. 1) and other glycated proteins containing Amadori adducts (apo B, fibronectin) did not react with A717 on Western blot. In ELISA, A717 exhibited a dose response reactivity with purified glycated albumin, in both unreduced and reduced constructs (Fig. 2). Since borohydride converts the ketoamine to glucitol-lysine, these findings indicated that the ketoamine structure is not exclusively necessary for recognition, which we have previously noted [27]. Preservation of reactivity after borohydride reduction is consistent with recognition of a linear form of the Amadori adduct, which is in equilibrium with the furanose configuration, with equilibrium shifted to the linear form upon borohydride reduction. None of the other tested purified glycated proteins reacted in ELISA with the A717 monoclonal antibody, consistent with the interpretation that the Amadori adduct epitope is encompassed in an albumin specific domain.

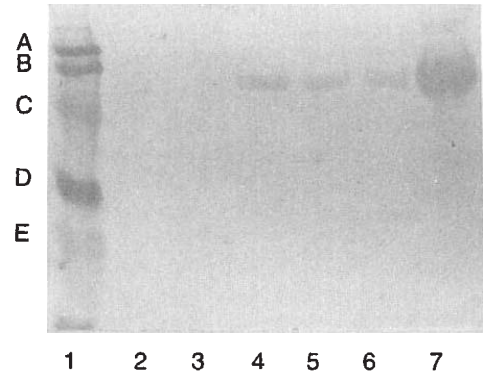

Fig. 1. *Specificity of A717 monoclonal antibody for glycated epitopes residing in albumin.* Electrophoresed samples were glycated albumin purified from normal plasma by sequential chromatography on Affi-gel Blue and PBA (lane 4), serum incubated for 4 days with 28 mM glucose (lanes 5 and 6), and albumin glycated *in vitro* for 7 days with 28 mM glucose (lane 7). Deglycated human albumin (pass-through from PBA) shown in lane 3. Glycated hemoglobin purified from human red cell lysates by sequential ion exchange and affinity (PBA) chromatography shown in lane 2. Molecular weight standard is shown on left (lane 1) where A = 106K (phosphorylase B); B = 80K (bovine serum albumin); C = 49.5K (ovalbumin); D = 32.5K (carbonic anhydrase); E = 27.5K (soybean trypsin inhibitor).

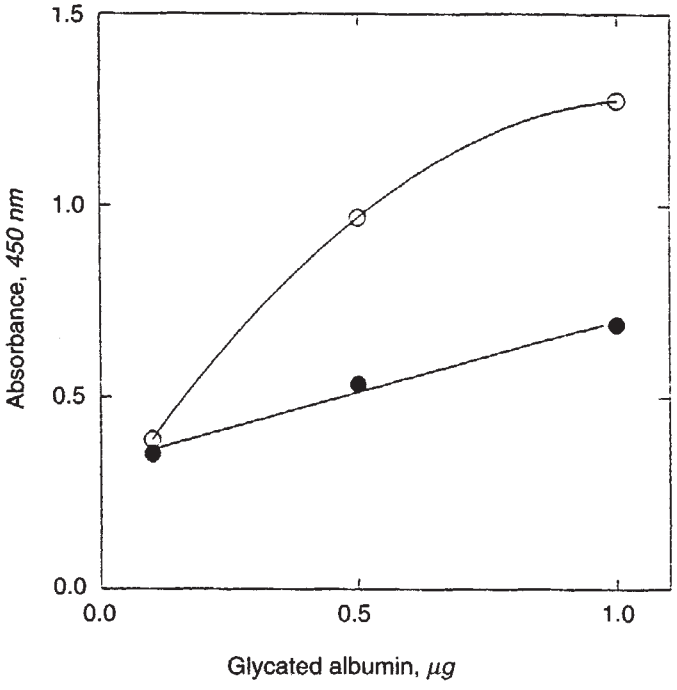

Fig. 2. *Reactivity of unreduced (●) and borohydride-reduced (○) glycated albumin in ELISA with monoclonal antibody A717.* Each point represents the mean of 6 observations; standard errors were less than 0.05 absorbance units for each point.

[^{3}H]-thymidine incorporation by murine mesangial cells in culture was stimulated in a dose response manner by the addition of normal human serum to media containing 5.5 mM glucose (Fig. 3). Mesangial cells proliferated more readily after short-term (48 hours) incubation in serum-free culture media containing 25 than 5.5 mM glucose (Fig. 3). We have previously

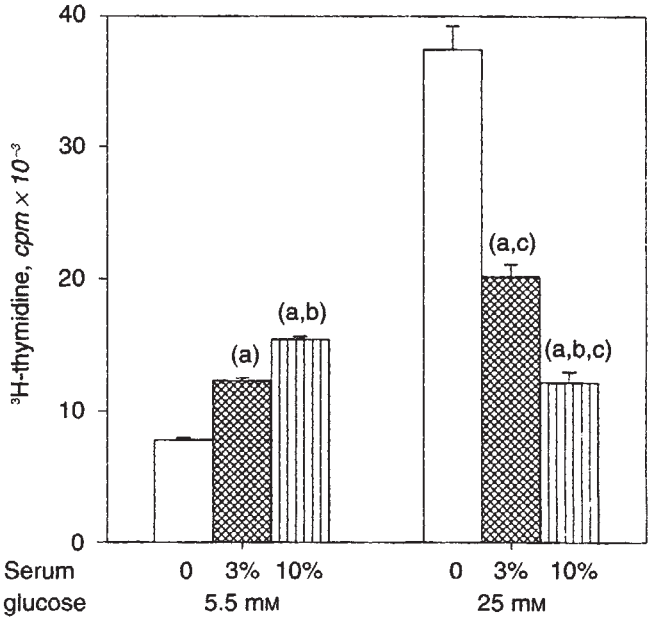

Fig. 3. *Effect of normal human serum in normal or elevated glucose concentration on the proliferation of mouse mesangial cells.* Each value represents mean ± SEM of four experiments in which transformed cells were pulsed for 8 hours with [^{3}H]-thymidine after culture for 48 hours in the presence of 5.5 mM or 25 mM glucose supplemented with the concentrations indicated of dialyzed human serum (HS). [a] $P < 0.05$ compared with 0% serum at same concentration of glucose; [b] $P < 0.05$ compared with 3% serum at same concentration of glucose; [c] $P < 0.05$ compared with same concentration of HS.

reported this finding, as well as the observations that incubation in 25 mM glucose for longer (72 hours) periods in serum-free media inhibits proliferation compared with 5.5 mM glucose, and that the high glucose effects are not reproduced by supplementation with mannitol [11]. Absence of mannitol effect (19.5 mM in 5.5 mM glucose) on cell proliferation was confirmed again in the present study. In contrast to the stimulated incorporation with normal serum in normal glucose concentration, the addition of 3 to 10% normal human serum to media containing 25 mM glucose resulted in a dose-dependent inhibition of thymidine incorporation, with 46% and 68% inhibition at 3% and 10% serum supplementation, respectively (Fig. 3).

These findings suggested that glycation of serum proteins during the 48 hour exposure to high glucose concentration might be responsible for the observed inhibition. Therefore, the effect of glycated serum on mesangial cell proliferation was directly examined, using two experimental approaches. First, in analogy with the procedure described by Crowely et al [7], cells were grown in plastic microtiter wells that had been precoated with normal serum or with four-day glycated serum by incubating the wells with 100 μl of the corresponding serum solution for three days at 4°C under sterile conditions. After aspiration of the serum and washing with PBS, incorporation by cells grown for 48 hours in serum-free media in the precoated wells was compared with that by cells grown in untreated wells. Precoating with normal serum resulted in modest but significant stimulation of thymidine incorporation, whereas precoating with glycated serum significantly inhibited proliferation (Fig. 4). The

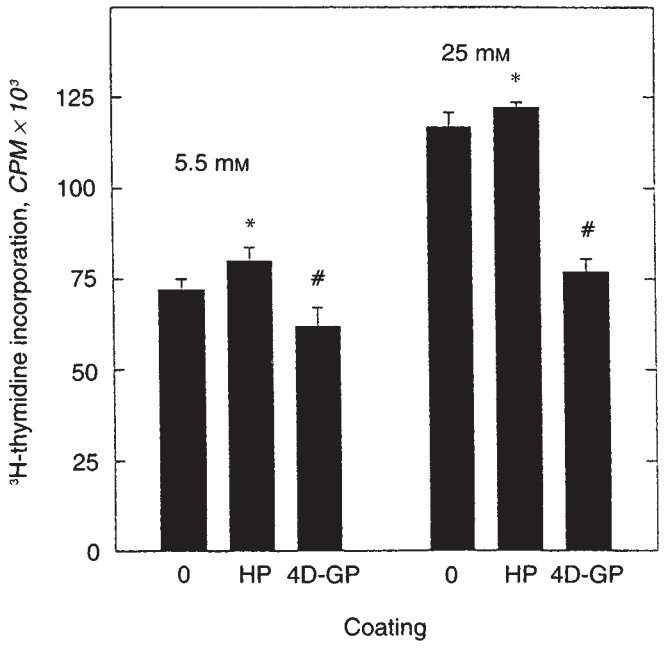

Fig. 4. *Effect of precoating of microtiter wells on mouse mesangial cell proliferation.* Each value represent mean ± SEM of 6 experiments in which transformed cells were pulsed for 8 hours with [^{3}H]-thymidine after culture for 48 hours in serum-free media containing 5.5 mM or 25 mM glucose in plastic microtiter wells that were untreated or had been precoated with normal serum (HP) or with 4-day glycated serum (4D-HP). *$P < 0.05$ compared with untreated microtiter wells; #$P < 0.05$ compared with microtiter wells precoated with normal serum.

Table 2. Effect of glycated serum in normal or elevated glucose concentration on proliferation of mouse mesangial cells in culture

Glucose	Supplement	cpm × 10^{-2}/well
5.5 mM	3% HS	120 ± 2
	10% HS	150 ± 2.5
	3% GS	100 ± 4.8[a]
	10% GS	57 ± 10[b]
25 mM	3% HS	202 ± 9
	10% HS	122 ± 8
	3% GS	200 ± 8
	10% GS	83 ± 9[a,b]

Each value represents mean ± SEM of four experiments in which cells were pulsed for 8 hours with [^{3}H]-thymidine after culture for 48 hours in the presence of 5.5 mM or 25 mM glucose supplemented with the concentrations indicated of dialyzed normal human serum (HS) or serum that had been incubated for four days with 28 mM glucose before dialysis (GS).

[a] $P < 0.05$ compared with same concentration of HS

[b] $P < 0.05$ compared with 3% GS

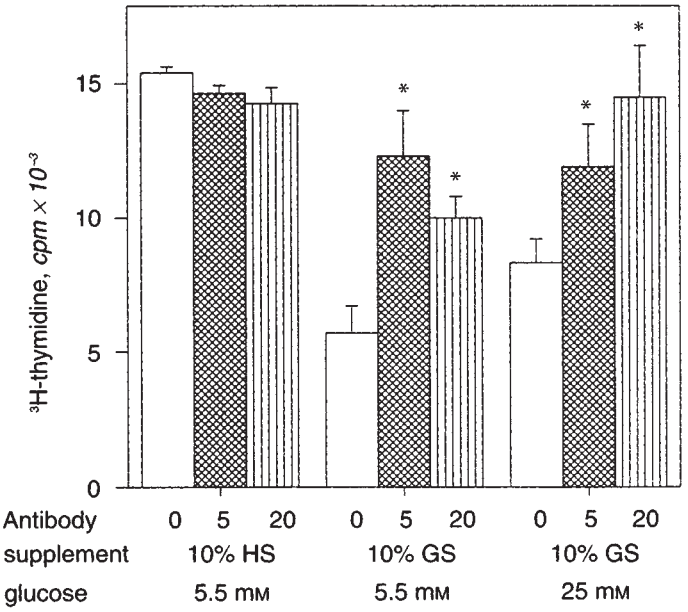

Fig. 5. *Effect of A717 monoclonal antibody on glycation-induced inhibition of mesangial cell proliferation.* Each value represents mean ± SEM of four experiments in which transformed cells were pulsed for 8 hours with [^{3}H]-thymidine after culture for 48 hours in the indicated glucose concentration supplemented with unincubated human serum (HS) or serum glycated by incubation for 4 days with 28 mM glucose (GS), without (0) or with A717 monoclonal antibody. *$P < 0.05$ compared with no antibody added.

inhibitory effect in cells cultured in normal (5.5 mM) glucose was modest (13% inhibition), but was striking in cells cultured in 28 mM glucose (34% inhibition). Second, incorporation by cells grown in untreated wells and incubated with media supplemented with glycated serum was compared with incorporation by cells incubated with media supplemented with normal serum. Serum glycated *in vitro* for four days dramatically inhibited proliferation in media containing 5.5 mM glucose (Table 2); this effect was dose-dependent on the concentration of glycated serum (17% inhibition and 62% inhibition at 3% and 10% glycated serum, respectively). Glycated serum in 25 mM glucose media exaggerated the high-glucose induced inhibition of mesangial cell growth. Relative to 10% normal serum in 5.5 mM glucose, 10% normal serum in 25 mM glucose inhibited incorporation by 19%; relative to 10% normal serum in high glucose, 10% glycated serum inhibited incorporation by 33% (Table 2). These changes in thymidine incorporation were accompanied by parallel changes in cell counts. In cells grown in 5.5 mM glucose for 48 hours, cell counts were reduced 8.8% in 3% glycated serum relative to 3% normal serum. In cells grown in 25 mM glucose, the reduction in cell number by glycated serum was 19.5% ($N = 4$ for each condition). Because culture in media containing glycated proteins in soluble phase more likely resembles the *in vivo* situation, where the mesangium is bathed by serum presented by the circulation, further experiments were conducted in media supplemented with glycated serum in the soluble phase.

Addition of A717 monoclonal antibody to mesangial cell cultures had no effect on basal incorporation in 5.5 or 25 mM glucose, and did not influence incorporation stimulated by normal serum in normal or elevated glucose. However, A717 restored proliferative activity in cells incubated with glycated serum and normal or elevated glucose concentration (Fig. 5). The A717 effect showed a dose response relationship in cultures incubated with 25 mM glucose. The observed effects were specific for the A717 antibody since the addition of 5 to 20 μg/ml of mouse or rabbit IgG that do not react with glycated albumin had no effect. Notably, the addition of A717 antibody to cells cultured with glycated serum restored thymidine incorporation to levels approximating those observed with cells cultured with

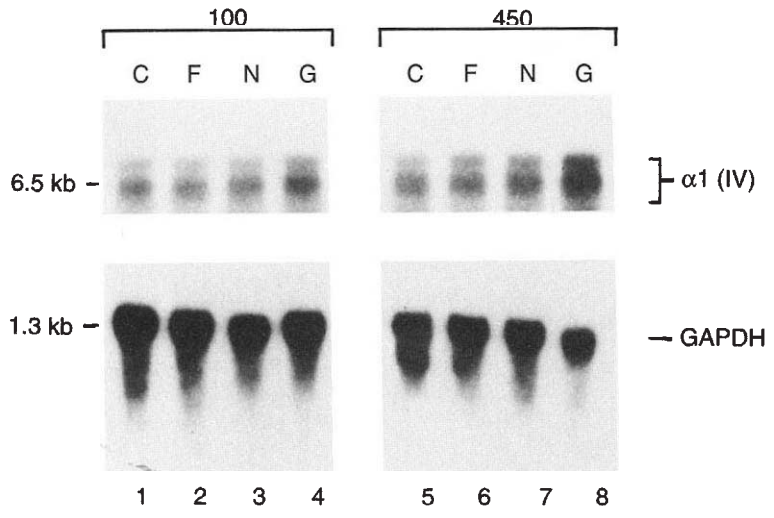

Fig. 6. *Representative autoradiograph of a Northern blot showing effect of culture medium composition on mRNA levels of the alpha 1 chain of Type IV collagen and GAPDH in non-transformed murine glomerular mesangial cells.* Cells were cultured for 72 hours in DMEM containing 100 (A, 5.5 mM) or 450 (B, 25 mM) mg/dl glucose. Lanes 1 and 5 (C), serum-free contro media; lanes 2 and 6 (F), 3% fetal calf serum; lanes 3 and 7 (N), 3% normal (unincubated) human serum; and lanes 4 and 8 (G), 5-day glycated human serum. The same filter was stripped and re-probed with GAPDH to control for equivalent RNA loading and transfer. Similar results were obtained in two separate experiments.

normal serum, and did not stimulate incorporation to levels greater than those observed in normal serum-supplemented cultures. Further, addition of A717 antibody to cells cultured with normal serum did not stimulate incorporation to levels greater than those observed without antibody. These findings militate against a non-specific antibody-mediated effect on proliferation through occupancy and activation of mesangial Fc receptors by antibody or by glycated albumin immune complexes. Response to A717 also was demonstrable with cell counts. In cells grown with 5.5 mM glucose plus 3% glycated serum, the addition of 5 μg/ml A717 monoclonal antibody increased cell counts by 17% compared with cell number in cultures grown under the same conditions but supplemented with 5 μg/ml mouse IgG that was not reactive with glycated albumin.

To determine if increased matrix synthesis accompanied the glycation-induced decrease in proliferation, which together are hallmarks of mesangial cell biology in diabetes, we examined Type IV collagen gene expression and secretion. Northern blot analysis using a cDNA probe for alpha$_1$-Type IV collagen demonstrated constitutive expression of the mRNA for collagen Type IV by non-transformed murine mesangial cells in serum free media containing 5.5 or 25 mM glucose, although Type IV collagen mRNA expression was stimulated in the high glucose concentration medium (Fig. 6). Supplementation with FCS or normal human serum had only a modest effect on Type IV collagen mRNA expression in either normal or elevated glucose concentration, but higher levels of expression were observed in FCS or NS-supplemented cells grown in high glucose concentration (Fig. 6). Cells grown in media supplemented with 3% glycated human serum expressed levels of Type IV collagen mRNA that were appreciably increased compared with those in cells grown in media supplemented with normal human serum. This effect was observed in both 5.5 and 25 mM glucose concentration, but was much more dramatic in high glucose concentration (Fig. 6). Densitometric scanning of the exposed films allowed calculation of relative RNA levels to adjust for small variations in RNA loading and transfer. Table 3 presents Type IV collagen to GAPDH ratios in RNA isolated from cells grown under the various culture conditions. Relative to serum-free media in 5.5 mM media glucose concentration, normal anc glycated human serum in 5.5 mM media glucose resulted in 45% and 60% increases in collagen mRNA, respectively; in 25 mM glucose concentration, normal serum increased collagen mRNA about threefold whereas glycated serum induced a ninefolc increase. Relative to serum-free media in 25 mM glucose concentration, normal serum increased collagen mRNA 23% whereas glycated serum induced an almost fourfold increase.

Table 3. Type IV collagen/GAPDH mRNA ratios

Glucose concentration	Supplement	Type IV: GAPDH
5.5 mM	none	1.00
	FCS	1.20
	NS	1.45
	GS	1.60
25 mM	none	2.35
	FCS	2.80
	NS	2.90
	GS	9.00

Serum supplements at 3%. Data obtained by densitometric scanning of autoradiographs of Type IV collagen and GAPDH hybridization assay, and represent the mean of two separate experiments under each condition. Abbreviations are: FCS, fetal calf serum; NS, normal human serum; GS, glycated human serum. Values represent ratios compared with no serum supplement in 5.5 mM glucose, assigned an arbitrary value of 1.00.

The changes in Type IV collagen mRNA expression accompanying exposure of mesangial cells to nonenzymatically glycated serum proteins were associated with parallel increases in Type IV collagen secreted into the media, which we and others have shown accounts for approximately 80% of the Type IV collagen produced by mesangial cells cultured in the presence of β-aminopropionitrile [6, 29]. Cells cultured during the experimental period in the absence of serum secreted detectable but very low levels of Type IV collagen into the medium. Supplementation with normal human serum resulted in a twofold increase in collagen secretion compared with non-supplemented media in normal or elevated glucose concentration. Supplementation with glycated serum significantly stimulated

Type IV collagen secretion, and this effect was exaggerated in high glucose concentration (Fig. 7). Relative to normal serum, glycated serum induced a 33% increase in 5.5 mM glucose concentration, and an 80% increase in 25 mM glucose concentration. This marked stimulation of Type IV collagen production by glycated serum in normal or elevated glucose concentration greatly exceeded the modest effect on total protein synthesis, measured by ^{3}H-leucine incorporation, under similar conditions. In cells grown in 5.5 mM glucose for 48 hours, 3% glycated serum increased tritiated leucine incorporation only 10% compared with incorporation in the presence of 3% normal serum (194 ± 18 vs. 176 ± 17 cpm/10^3 cells, respectively; N = 4 each condition; P = NS). In cells grown in 25 mM glucose, the increase was 16% (from 272 ± 21 to 316 ± 18 cpm/10^3 cells; N = 4 each condition; $P < 0.05$). Supplementation with mannitol (19.5 mM in 5.5 mM glucose) was without effect on collagen IV production or leucine incorporation [11].

Addition of A717 antibody had no effect on collagen secretion by cells cultured in the presence of normal human serum, but prevented the increased secretion associated with culture in the presence of glycated human serum (Fig. 7). The prevention of glycated serum-induced increase in collagen production was specific for the A717 antibody, since the addition of mouse or rabbit IgG not containing antibodies to glycated albumin had no effect. A717 or other IgG added to cells cultured in the absence of serum, whether in low or high glucose concentration, also had no effect. Notably, addition of A717 to cells cultured with glycated serum restored collagen secretion to levels observed with cells cultured with normal serum, and did not inhibit production to levels lower than those observed in normal serum-supplemented cultures. Further, addition of A717 antibody to cells cultured with normal serum did not depress collagen synthesis to levels lower than those observed without antibody or to levels observed in cells grown in the absence of serum. As discussed above, these findings indicate that the reversal of the stimulatory effect of glycated serum was due to blocking of glycated epitopes in albumin and not a non-specific Fc-mediated signal inhibiting collagen synthesis.

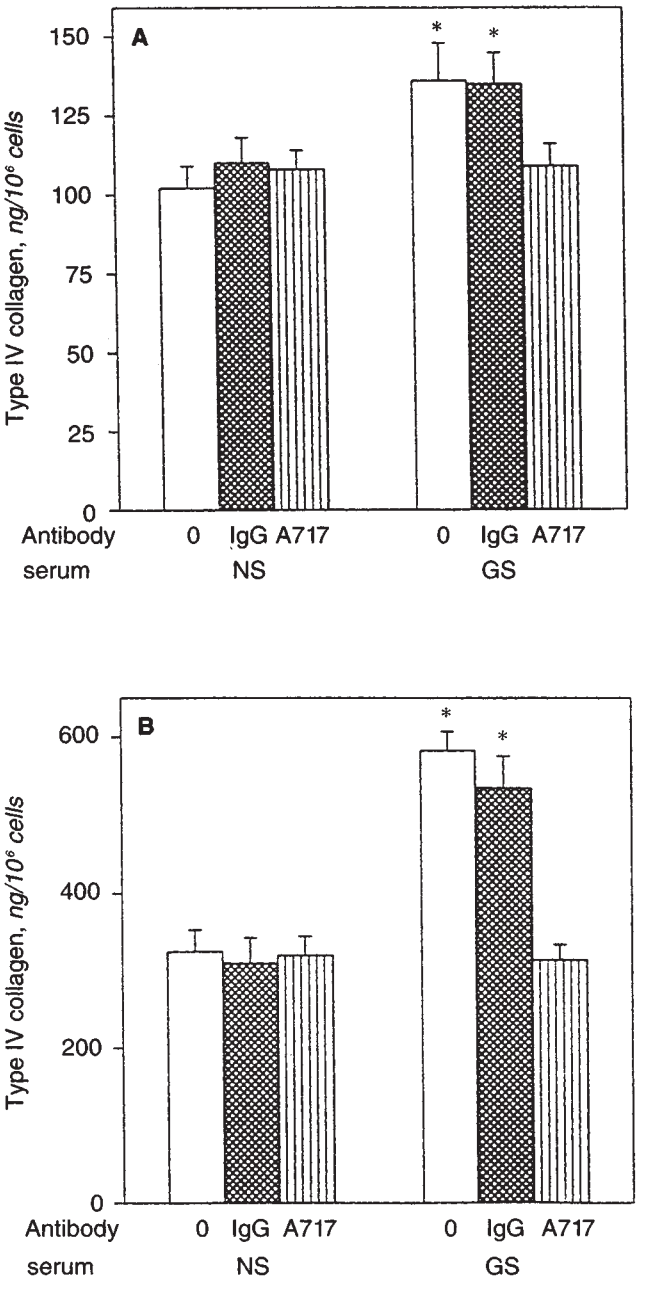

Fig. 7. *Type IV collagen secretion by non-transformed mouse mesangial cells cultured in media containing 5.5 mM (A) or 25 mM (B) glucose in the presence of 3% normal (NS) or glycated (GS) human serum, without (open bars) or with the addition of IgG not reactive with glycated albumin (hatched bars) or A717 monoclonal antibody (lined bars).* Results represent mean ± SEM of four separate experiments under each culture condition. *$P < 0.05$ compared with NS without IgG or A717 added.

Discussion

Previous studies have examined the effect of glucose *per se* on proliferation of mesangial cells, reporting variable results including stimulation, no effect, or inhibition, depending on cell type, duration of exposure to glucose, or culture conditions [11, 29, 36–39]. An effect of nonenzymatic glycation of serum proteins and of Amadori adducts in a particular serum protein on cell proliferation has not been demonstrated previously. Crowley et al found that plating rat mesangial cells onto extracellular matrix that had been elaborated by the cells in culture and was modified by incubation with glycoaldehyde (50 mM) or G-6-P (200 mM) reduced [^{3}H]-thymidine incorporation relative to incorporation by cells plated onto unmodified matrix [7]. However, the elaborated matrix contained laminin, fibronectin and collagen but no albumin; glycoaldehyde is a highly cross-linking glycation product; and both G-6-P and glycoaldehyde modified matrices were highly fluorescent, indicating extensive formation of AGE products. The inhibition of cell proliferation in that study was therefore ascribed to glycation-induced cross-linking of matrix components, which was consistent with the investigators' finding that inhibition appeared proportional to the degree of cross linking assessed by fluorophore analysis.

The present results, in contrast, demonstrate that glycated serum proteins without cross-links or AGE fluorescence inhibit cell growth and stimulate Type IV collagen production, and that these effects are due to Amadori adducts. Four considerations

support this interpretation. First, incubation of serum was kept to four to five days and performed under conditions known to yield Amadori and not AGE modification [25, 26, 35]. Second, the A717 antibody was raised against glycated albumin that was purified from normal plasma, representing albumin glycated *in vivo*, and did not contain AGE-modified albumin. Third, the antibody specifically immunoreacts with glycated lysine epitopes in albumin, does not recognize nonglycated albumin and does not immunoreact with protein in the phenylboronate (PBA) pass-through after application of albumin prepared from human plasma or of albumin glycated *in vitro*. Even if AGE products of serum glycoalbumin are formed *in vivo* or *in vitro* after four to five days of incubation, they would be present in the PBA pass-through since co-planar cis-hydroxyl groups are required for binding to PBA. AGE products, which are irreversibly formed, do not have this configuration. The ability of the A717 antibody to react with unreduced or borohydride-reduced glycoalbumin is consistent with recognition of a fructosyl adduct, with equilibrium shifted to linear configuration by borohydride reduction. Further, the antibody does not immunoreact with other proteins, whether glycated or not, strongly suggesting that glycated albumin is responsible for the observed effects. This contrasts with antibodies raised against AGE products, reported to prevent cell biology effects of AGE-modified proteins, which recognize immunologic epitopes that appear to be common to many different proteins after incubation for prolonged periods with high concentrations of reducing sugars [20].

Glycated serum containing increased concentrations of glycated albumin stimulated collagen production relative to normal serum containing normal concentrations of glycated albumin. An effect of A717 antibody was only observed in cells cultured with glycated serum; the antibody had no effect on collagen synthesis by cells cultured with fetal calf or normal serum. Abrogation of the glycated serum effect by A717 antibody indicates that (increased) glycated albumin is responsible for the increased collagen synthesis relative to normal serum. If occupancy of the Fc receptor by glycated albumin immune complexes caused (non-specific) inhibition of collagen synthesis, such an effect would have been observed in cells cultured in normal serum in the presence of antibody.

The results presented in these experiments are the first to demonstrate that Amadori adducts in nonenzymatically glycated serum proteins induce increases in mesangial cell collagen Type IV gene expression. In these experiments, we used mesangial cells cultured in media containing glycated serum, which resembles the *in vivo* situation where the mesangium is bathed in serum presented by the circulation. We believe that culture in media containing glycated proteins in soluble phase, in amounts representative of those found in diabetic serum and in Amadori configuration, more likely represents the *in vivo* milieu than does culture of cells in plates onto which proteins extensively modified by advanced glycation end (AGE) products have been immobilized or in medium to which AGE-modified bovine albumin in excessive amounts is added [7, 8]. Supplementation of culture media with normal or glycated serum also is more representative of *in vivo* physiology than is culture in serum-free media, and the observed stimulation of collagen gene expression by glycated serum proteins adds to our understanding of hyperglycemia-related pathophysiologic changes in mesangial cells. Further, we suggest that the stimulation of Type IV collagen gene expression and production by glycated serum that was observed with physiologic glucose concentration and was accentuated with high glucose concentration approximates the *in vivo* situation in which there are periods of normoglycemia and hyperglycemia. It is interesting to note that the glycation-induced changes in proliferation, collagen secretion, and collagen gene expression were observed in normal glucose concentration and were accentuated in elevated glucose concentration. Sabbatini et al also found that acute hyperglycemia caused an impressive exaggeration of the glycated serum-induced hyperfiltration in non-diabetic rats [25].

We recognize that caution should be exercised in extrapolating data from tissue culture studies to the *in vivo* state. Nevertheless, we believe that these results, taken in context with other studies, are highly relevant to the pathogenesis of diabetic nephropathy. Glycated albumin, which is increased in diabetic serum, is known to enter the renal glomerulus *in vivo*, where it is taken up more avidly than nonglycated albumin [15, 18, 21–24]. Nondiabetic mice injected with glycated plasma proteins have developed glomerular basement membrane thickening, endogenous albumin binds to the glomerular basement membrane, and there is widespread connective tissue accumulation of glycated protein in diabetes [40–43]. Enhanced uptake of glycated albumin by glomerular mesangial and epithelial cells is accompanied by adverse metabolic changes [24]. The glycated protein-induced inhibition of cell proliferation and stimulation of collagen production parallel effects that have been observed with certain important growth factors such as TGF-β, which has been shown to be anti-mitogenic and pro-sclerotic in a variety of cells, including mesangial cells [11, 44]. Increased elaboration of matrix macromolecules by mesangial cells is considered to be an early and characteristic lesion in diabetic nephropathy; decreased cellularity as mesangial matrix accumulates and diversion of growth potential to synthesis of matrix macromolecules, as shown in these experiments, is consistent with pathologic findings.

The mechanisms by which glycated serum proteins lead to inhibition of proliferation and stimulation of collagen gene expression and collagen production are currently unknown, but the findings suggest that there might be cell binding proteins that specifically interact with Amadori adducts. This possibility also was suggested by the studies of Predescu et al, which demonstrated that glycated albumin can bind to endothelial cells as a bifunctional ligand, with a plasma membrane site that binds as a lectin glucose in glycated albumin and a plasmalemmal site that recognizes domains in the albumin molecule [45]. It is further supported by our recent identification in aortic endothelial cells of glycated albumin-binding polypeptides [46]. Thus, it is possible that binding of this circulating glycated protein to a plasma membrane site triggers receptor-induced events, analogous to the many other models of ligand-receptor interaction. Alternatively, glycated albumin may, after internalization [45], be responsible for the observed consequences on mesangial cell biology.

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